Genetic variation in FOXO3 is associated with reductions in inflammation and disease activity in inflammatory polyarthritis

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ABSTRACT

Background: Genetic variation in *FOXO3* (tagged by rs12212067) has been associated with a milder course of rheumatoid arthritis (RA) and shown to limit monocyte-driven inflammation through a TGFβ1-dependent pathway. This genetic association, however, has not been consistently observed in other RA cohorts. We sought to clarify the contribution of FOXO3 to prognosis in RA by combining detailed analysis of non-radiographic disease severity measures with an *in vivo* model of arthritis.

Methods: Collagen-induced arthritis, the most commonly used mouse model of RA, was used to assess how Foxo3 contributes to arthritis severity. Using clinical, serological and biochemical methods, the arthritis that developed in mice carrying a loss-of-function mutation in *Foxo3* was compared with that which occurred in littermate controls. The association of rs12212067 with non-radiographic measures of RA severity, including CRP, Swollen Joint Count, Tender Joint Count, DAS28 and the HAQ score were modelled longitudinally in a large prospective cohort of early RA patients.

Results: Loss of Foxo3 function resulted in more severe arthritis *in vivo* (both clinically and histologically) and was associated with higher titres of anti-collagen antibodies and IL-6 in blood. Similarly, rs12212067 (a SNP that increases *FOXO3* transcription) was associated with reduced inflammation – both biochemically and clinically – and with lower RA activity scores.

Conclusions: Consistent with its known role in restraining inflammatory responses, FOXO3 limits the severity of *in vivo* arthritis and, through genetic variation that increases its transcription, is associated with reduced inflammation and disease activity in RA patients – effects that would lead to lesser radiographic damage.

Key words: genetics, *FOXO3*, rheumatoid arthritis, outcome, disease activity

INTRODUCTION

Like most autoimmune and inflammatory conditions, the course of rheumatoid arthritis (RA) is unpredictable and highly variable between patients. While current guidelines advocate early and aggressive treatment for all patients to avoid irreversible joint damage and disability, this strategy will undoubtedly result in the overtreatment of patients with mild disease or those in whom the disease would have spontaneously entered remission. Accordingly, such patients are currently exposed to the risks and side-effects of unnecessary immunosuppression. To overcome this limitation, a personalised management strategy would be necessary, which in turn would rely on having a reliable method of predicting disease outcome. Currently, however, the predictive value of clinical or serological markers is insufficient to guide treatment decisions, and accordingly there is a clear and well-recognised need for predictive biomarkers.

Genetics has been shown to play an important role in the development of RA through a series of large genome-wide association studies,[1-3] but its potential role in determining disease course following diagnosis has been less rigorously studied – even though radiographic outcome has been proposed to be partially genetically determined.[4] Indeed, while many studies have reported genetic associations with outcome, virtually none of these outside the HLA reach genome-wide significance, nor have they always been replicated.[5-9] We previously demonstrated that a SNP in *FOXO3A* (rs12212067: T>G) was associated with prognosis in several TNFα-driven diseases and that this variant led to altered production of pro- and anti-inflammatory cytokines by monocytes through a TGFβ1-dependent pathway [10]. In RA specifically, we showed that minor allele carriage at rs12212067 – which reduced TNFα, IL-6, IL-1β and IL-8 production and increased IL-10 production – was

associated with a milder course of disease (i.e. less joint damage over time) in large and well phenotyped cohorts of patients with early disease. Since that report, which also demonstrated that rs12212067 was associated with outcome in Crohn's disease and malaria, several smaller studies have attempted to replicate these associations. In Crohn's disease[11] and malaria,[12] the reported associations have been independently replicated, although in RA a meta-analysis of five smaller cohorts from the US and Europe failed to detect the same association signal.[13] This situation is not unusual, and in fact is similar to that which occurred in the early GWAS era when some of the first associations – many of which have since been replicated several times – were initially questioned by negative follow-up studies in small and consequently underpowered cohorts.[14-16]

Here, we sought to better understand what contribution FOXO3 might make to the clinical outcome of RA. To do this we first investigated whether a role for FOXO3 in altering arthritis severity was biologically plausible by examining the contribution of FOXO3 to the severity of immune-mediated arthritis *in vivo*. We then chose to examine a broader range of arthritis severity markers in a large cohort of patients with early RA and inflammatory polyarthritis to determine whether other associations might be present that would support (or indeed refute) the association of rs12212067 with outcome in RA.

METHODS

Collagen-induced arthritis

To study the role of FOXO3 in immune-mediated arthritis *in vivo*, we used the collagen-induced arthritis mouse model – the most commonly used animal model of RA – in which arthritis is induced by immunisation with emulsified Type II Collagen in Complete Freund's Adjuvant (50 µl, 4 mg/ml, intradermal). We compared the severity of the resulting arthritis in C57BL/6 mice harbouring a missense mutation in the highly conserved Forkhead DNA-binding domain that abrogates the function of Foxo3 (Foxo3a $^{MommeR1/MommeR1}$ mice; from here termed Foxo3a-/-),[17] with that which developed in heterozygous (Foxo3a+/-) and wild-type (Foxo3a+/+) littermates (all 8 weeks old). Arthritis severity was assessed using a standard scoring system[18] and serum anti-collagen antibodies (Chondrex), IL-6 and TNFα levels (R&D Systems) were assessed at day 14 by ELISA. All experiments were performed according to the regulations of the UK Home Office Scientific Procedures Act (1986).

Patient cohorts and genotyping

To further investigate the relationship between rs12212067 (a non-coding SNP within *FOXO3*) and severity in RA, we used the Norfolk Arthritis Register (NOAR), a primary care-based inception cohort of 4293 patients with early inflammatory polyarthritis (IP) who were followed prospectively from disease onset for up to 20 years[19] (Supplementary Table 1). IP patients who satisfied the 1987 ACR criteria – applied cumulatively over the first 5 years of follow-up – were called RA patients (n = 2537 Supplementary Table 1). Where available, we analysed the association of

genotype at rs12212067 with a range of RA severity indices, including (1) radiographic measures (Larsen score,[20] modified Larsen score and presence of erosions – defined as a cortical break ≥2mm), (2) biological measures (C-reactive protein [CRP]), (3) patient-reported outcomes (Health Assessment Questionnaire [HAQ] score) and (4) disease activity scores: Swollen Joint Count (SJC), Tender Joint Count (TJC) and Disease Activity Score based on 28 joints (DAS28) - calculated using 3 variables (SJC, TJC and CRP). Genotyping of rs12212067 was performed on a custom-designed genotyping chip (Illumina Immunochip) or using Sequenom MassArray technology. Quality control procedures for ImmunoChip data were as described in the original report.[2] Ethical approval for the NOAR study was given by the Norwich Research Ethics Committee and all patients gave informed consent.

Statistical analysis

In order to maximize power for the studies of RA or IP outcome, we incorporated multiple records per patient over time and considered all measures of disease outcome (or activity) as longitudinal continuous variables. All analyses were adjusted for intra-individual correlation, and a dominant model of association was used (in part due to the low frequency of minor allele homozygotes). To provide a positive control, we also analysed the shared epitope (SE), a five amino acid sequence motif in the HLA-DRβ chain that is known to be associated with a variety of measures of severe rheumatoid arthritis, using the same method. Presence or absence of the SE was determined experimentally using the reverse dot-blot method as described previously.[21] We used Generalized Linear Latent and Mixed Modelling (GLLAMM) for measures of radiographic outcome,[22] zero-inflated negative binomial regression

(ZINB) for CRP,[23] and quantile (median) regression for the HAQ score, SJC, TJC and DAS28. More details are provided in Supplementary methods. Because the directions of effect of the shared epitope and the *FOXO3A* SNP are already known, one-tailed p-values are reported, as in the original study [10]. Adjustment for multiple testing and treatment strategies was performed where indicated. Treatment was integrated in the model as a time-dependent dummy variable for the presence of treatment with any disease modifying anti-rheumatic drug (DMARD) at every follow-up. The proportion of the variance of a specific trait explained by the *FOXO3A* SNP was calculated as follows: the model was run with and without the SNP; for each model, the correlation coefficient between the observed and predicted values was calculated; the difference between the squares of the correlation coefficients was used to quantify the proportion of the variance explained by the SNP. These analyses were performed using Stata version 11 / 13 software (Copyright 1985-2011 StataCorp LP, Texas, USA).

In the collagen-induced arthritis experiments, comparison of the arthritis score between *Foxo3*-/-, *Foxo3*+/- and *Foxo3*+/+ was performed using a two way analysis of variance (ANOVA) and comparison of biochemical or serological parameters between genotypes was performed using a Wilcoxon matched pairs signed-rank test. One tailed p values are presented in view of the specific hypothesis being tested. These analyses were performed using GraphPad Prism v6.04 (GraphPad Software, La Jolla California USA, www.graphpad.com).

RESULTS

To investigate how altered FOXO3 function might influence the course of autoimmune arthritis *in vivo*, we first assessed the severity of collagen-induced arthritis in mice carrying a missense mutations in the highly conserved Forkhead DNA-binding domain of *Foxo3*, known to abrogate its function. We showed that carriage of the Foxo3 null allele was associated with a more severe arthritis. This was apparent from both the significantly higher arthritis scores (Figure 1A) and from joint histology, which demonstrated increased synovial expansion and cellularity and tissue oedema (Figure 1B). In addition to assessing the clinical severity of arthritis, we also sought to compare the titres of anti-collagen antibodies that were formed, as this enables a more direct evaluation of the immune response against type II collagen. These antibodies are also known to target epitopes within type II collagen that are shared by rheumatoid arthritis antibodies.[24] We demonstrated that higher levels of anti-collagen antibodies were present in mice with impaired or abrogated Foxo3 function (Figure 1C). We also assessed the production of IL-6 and TNFα at day 14, pro-inflammatory cytokines that are known to be important in the pathogenesis of CIA, and observed a non-significant trend to higher IL-6 levels in Foxo3-/- mice compared to either wild type or heterozygous littermates ($P = 0.06$, Figure 1D). Serum TNFα was not detectable at this time point (data not shown).

In light of these results, and our previous work that demonstrated an association between genotype at rs12212067 and the radiographic course of RA, we elected to examine other indices of disease severity in a large prospectively collected cohort of early RA patients. First we considered other radiographic measures as it has recently been suggested that the number of eroded joints may constitute a more accurate measure of joint damage [25]. We confirmed that irrespective of the

measure used, carriage of the minor allele at rs12212067 was associated with lower radiographic damage over time (i.e. lower Larsen score, lower modified Larsen score and fewer erosions, Table 1). In other words, patients with a milder disease course were more likely to carry the minor allele of rs12212067 than patients with a more severe disease course (the average minor allele frequency of rs12212067 in NOAR is 9.6%). The effect of *FOXO3A* rs12212067 on the Larsen score remained significant after adjustment for treatment (effect size equal to a reduction of 1.50 Larsen units for the carriage of the G allele, p-value = 0.009) and was independent of the SE in a bivariate analysis (p-value = 0.002; Table 1). However, although this association was significant, the proportion of the variance of the Larsen score explained by the carriage of rs12212067 was low (0.07%).

Given these data, and the TGFβ1-driven inflammatory pathway that FOXO3 has been shown to regulate [10], we postulated that the effect of *FOXO3A* on radiographic outcome might be mediated by a reduction of inflammation, and not by a direct effect on the bone, as has been suggested for other genetic markers of RA severity.[5] We therefore examined the entire NOAR cohort to determine whether there was an association between rs12212067 genotype and non-radiographic measures of inflammation, disease activity or outcome (including CRP, SJC, TJC, the HAQ score and DAS28). Data availability and the frequency and duration of follow-up for these different measures varied and are shown in Supplementary Table 2. We observed significant associations of rs12212067 genotype with DAS28, SJC and CRP and a nominally significant association with HAQ score (p = 0.013, although the stringent bootstrap correction for inter-individual correlation meant that the corrected P value was 0.11). Notably, there was also a consistent direction of effect across all of the variables, with minor allele carriage at rs12212067 generally

associating with indices of milder disease, and with effect sizes that were of a comparable magnitude to those that were associated with the SE (Tables 1 and 2). However, the proportion of the phenotypic variance explained by the carriage of rs12212067 remained low for non-radiographic measures of disease outcome (the proportion of the variance of DAS28 explained by the SNP only was 0.09%).

The anticyclic citrullinated peptide (anti-CCP) antibody status is a strong and well established predictor of disease severity [33] and the effect of the SE on RA severity is mainly mediated by anti-CCP [22, 34]. In order to investigate the influence of anti-CCP status on the association of *FOXO3A* rs12212067, we adjusted the effect of rs12212067 for anti-CCP status and also performed a stratification analysis by anti-CCP status (**Table 3**). Although the effect of the SE on disease severity and activity disappeared completely after adjustment or stratification, the effect of rs12212067 on Larsen score or DAS28 remained significant irrespective of anti-CCP status (the effect size adjusted for anti-CCP was equal to a reduction of 1.26 Larsen units for the carriage of the G allele (95% confidence interval: -2.49; -0.03), p-value = 0.023; and the OR for DAS28 was equal to 0.78 (95% confidence interval: 0.67;0.90), p-value = 4.3E-04).

	Shared epitope		*FOXO3A* (rs12212067)	
	Effect size (95% CI)	P value	Effect size (95% CI)	P value
Larsen score (unadjusted)	+ 1.42 (0.50;2.34)	0.0013	- 1.50 (-2.73;-0.26)	0.0089
Larsen score (adjusted for the shared epitope)			- 1.88 (-3.15;-0.60)	0.0020
Larsen score (adjusted for treatment)			- 1.50 (-2.75;-0.25)	0.0094
Modified Larsen score	+ 1.51 (0.66;2.35)	0.00024	- 1.65 (-2.80;-0.50)	0.0025
Number of erosive joints	+ 0.36 (0.17;0.54)	0.00012	- 0.29 (-0.53;-0.06)	0.0073

Table 1. Association of *FOXO3A* and the shared epitope with radiological measures of disease outcome

Modified Larsen score: obtained by recalculating the Larsen score using the presence of joint space narrowing (in the absence of any other modification) as “0” instead of “1”, in order to reduce potential misclassification due osteoarthritis.

Effect size refers to change in Larsen score units or numbers of erosive joints respectively.

CI, Confidence Interval

	Shared epitope		*FOXO3A* (rs12212067)	
	Odds ratio (95% CI)	P value	Odds ratio (95% CI)	P value
HAQ score	1.15 (1.06-1.25)	0.00019	0.94 (0.84-0.96)	0.11
DAS28	1.19 (1.02-1.38)	0.013	0.78 (0.66-0.93)	0.0029
SJC	1.22 (1.05-1.42)	0.0052	0.86 (0.73-1.03)	0.048
TJC	1.08 (0.80-1.45)	0.3	0.84 (0.59-1.20)	0.17
CRP	1.38 (1.09-1.72)	0.0032	0.74 (0.56-0.97)	0.016

Table 2. Association of *FOXO3A* and the shared epitope with non-radiological measures of disease outcome or activity

For outcome measures modelled with quantile regression, the following is reported: Odds ratio (e raised to the power of the observed coefficient), normal-based 95% Confidence Interval (CI), Bootstrap one-tailed p-value.

For CRP (modelled with ZINB) results from the categorical (inflate) part of the model are reported: Odds ratio (e raised to the power of the observed coefficient for being non-zero), 95% Confidence Interval (CI), robust p-value.

HAQ: Health Assessment Questionnaire. DAS28: Disease Activity Score based on 28 joints (SJC, TJC, CRP). SJC: Swollen Joint Count. TJC: Tender Joint Count. CRP: C-reactive protein

Table 3. Adjustement for and stratification by anti-CCP status.

	Shared epitope						***FOXO3A* (rs12212067)**					
	Anti-CCP positive		**Anti-CCP negative**		**Adjusted for anti-CCP status**		**Anti-CCP positive**		**Anti-CCP negative**		**Adjusted for anti-CCP status**	
	Effect size (95% CI)	P value	Effect size (95% CI)	P value	Effect size (95% CI)	P value	Effect size (95% CI)	P value	Effect size (95% CI)	P value	Effect size (95% CI)	P value
Larsen score	+ 1.05 (-1.45;+ 3.54)	0.205	+ 0.23 (-0.61;+1.07)	0.295	+ 0.16 (-0.80;+1.11)	0.373	-1.82 (-4.90;+ 1.26)	0.123	-0.98 (-1.96;+ 0.01)	0.026	-1.26 (-2.49; -0.03)	0.023
DAS28	1.13 (0.85;1.50)	0.205	1.08 (0.90;1.28)	0.206	1.10 (0.95;1.27)	0.104	0.78 (0.60;1.02)	0.033	0.80 (0.66;0.96)	0.010	0.78 (0.67;0.90)	4.3E-04

The association studies presented in **Table 1** for the Larsen score in RA and in **Table 2** for DAS28 in inflammatory polyarthritis have been performed again either by adjusting for anticyclic citrullinated peptide (anti-CCP) antibody status (column 3 and 6) or by stratifying by anti-CCP status, i.e. the analysis was restricted exclusively to anti-CCP positive (column 1 and 4) or anti-CCP negative disease (column 2 and 5). Since the effect of the SE is almost completely mediated by anti-CCP, it disappears completely. However, the adjustment has no major influence on the association of *FOXO3A* rs12212067. Stratification decreases sample size and therefore power, which is likely to explain the lack of significance in the smaller anti-CCP positive group for the Larsen score. Overall, these results indicate that the biological pathways mediating the effect of the SE and *FOXO3A* are different. CI, Confidence Interval; p-values are one-tailed; dimensions and interpretation of effect sizes are explained in the legend of **Table 1 and 2**.

DISCUSSION

FOXO3 is a transcription factor that has been linked to the regulation of immune responses using systems biology[26] and knockout mouse models[27] and which has been shown to be overexpressed in blood and synovial leukocytes in RA.[28] We have previously reported that minor allele carriage at rs12212067, a non-coding SNP in *FOXO3*, is associated with a milder course of several TNFα-driven diseases, including RA, and have described a FOXO3-dependent pathway that is regulated by this genetic variant and which – via TGFβ1 induction – modulates inflammatory responses in monocytes.[10] This genetic association, however, was not detected in a follow-up study by van Steenbergen and colleagues, which included 5 smaller RA cohorts and a mixture of prospective and retrospective patients, and of early and established RA.[13] A non-significant trend to a protective effect of the minor allele at rs12212067 – consistent with our results – was, however, observed in their meta-analysis, and particularly in the cohorts of early RA patients for whom multiple sets of radiographs were available.[13] Nonetheless, given these apparently contradictory reports,[10, 13] we sought to better understand what role, if any, FOXO3 plays in influencing disease course in RA.

We first sought to establish whether a contribution of FOXO3 to the course of immune-mediated arthritis was biologically plausible, given that genetic associations alone do not establish biological relevance to disease. Indeed, even though we had previously identified an inflammatory pathway that is modulated by genetic variation at rs12212067, this does not definitively prove that any association with radiographic outcome in RA is due to effects on inflammation. We therefore examined the role of Foxo3 in immune-mediated arthritis *in vivo* using an animal model that has previously been extensively and successfully used to delineate pathogenic

mechanisms in RA and test new therapies.[18] Based on the results of these experiments, we then used detailed phenotypic data from the largest cohort of early RA patients worldwide to assess for associations with other measures of inflammation and disease severity. We demonstrate that altered Foxo3 function does modulate the severity of autoimmune arthritis *in vivo*, and identify genetic associations with several other indices of inflammation and disease severity in RA patients. Together, these results strengthen the previously reported association of FOXO3 with outcome in RA. Moreover, these data suggest that rather than having a direct effect on bone, the contribution of FOXO3 to outcome in RA is likely to be by modulating inflammation (e.g. CRP) and disease activity (e.g. SJC, DAS28) which would then lead to differences in radiographic outcome (e.g. Larsen score, erosions). However, the effect of FOXO3 is independent of the SE and of the presence of anti-CCP antibodies, which is consistent with two different mechanisms of action for these two different genetic markers. Importantly, the size of the effects on radiographic damage and DAS28, while statistically significant, are unlikely to be clinically useful in isolation (e.g. for applications such as predicting disease outcome), as the proportion of the phenotypic variance explained by the FOXO3 SNP only is below 0.1% (0.07% for Larsen score and 0.09% for DAS28). This does not preclude, however, the inclusion of this SNP in a model that could incorporate several predictive factors (e.g. genetic, demographic, clinical and environmental). In the future, such a model could comprise a set of several hundreds of SNP of small effect sizes. With current technological advances and the commercialization of high throughput (chip-based) genotyping platforms for clinical applications, this approach will become a realistic diagnostic/prognostic option. Moreover, irrespective of the utility of this SNP for predicting disease course, by studying the functional effects of

such associations, it may be possible to uncover previously unappreciated pathways that are amenable to pharmacological intervention, and so develop better treatments. Such a discrepancy between the effect size of an associated SNP and the therapeutic potential of targeting the underlying biology has already been observed in cardiovascular medicine, where genetic variants in *HMGCR* are associated with very modest changes in serum LDL (~5%, [29]) but the protein product of this gene, HMG CoA reductase, is the pharmacological target of the most effective drug treatment for hypercholesterolaemia[30] (statins). An important caveat, however, is that the associations of rs12212067 with the indices of RA severity described herein have not yet been replicated, even though these indices were selected because of the *a priori* hypothesis that minor allele carriage at rs12212067 would associate with milder disease. Accordingly, these associations should be examined further in larger and appropriately-powered replications cohorts.

Since the advent of GWAS, it has been commonplace for the reporting of new genetic associations to be followed by several smaller, and often underpowered, studies that attempt to replicate the reported associations, with varying degrees of success. Here, we confirm that FOXO3 plays an important role in the determining the course of immune-mediated arthritis *in vivo* and detect associations between genotype at rs12212067 and a range of RA severity measures. It is therefore important to consider why this association was not detected in a previous follow-up study.[13] One possibility is that the genetics and biology of disease outcome are genuinely different between the populations studied. However, given that both study populations were derived from the same ethnic ancestry, this seems less likely and other possibilities need to be considered. An alternative explanation is that the relatively small size of the individual cohorts in the replication study[13] may have

limited their power to detect any effect. This is likely to have been further hampered by the considerable inter-cohort heterogeneity, both in terms of clinical phenotype (i.e. early vs. established RA) but also in terms of the available radiographic data. For example, in 50% of the cases only a single set of radiographs were available. This meant that progression could not be assessed in a uniform way across all cohorts, being directly measured in some, and extrapolated based on assumed linear disease progression in others, an assumption that is unlikely to be valid for most patients.[31] Standardised phenotype definitions are critical for multi-centre studies to prevent introducing site-based bias(es) and to ensure that the final meta-analysis is interpretable.[32] Accordingly, we would contend that rather than disproving a role for FOXO3 in the outcome of RA, this negative study actually re-emphasises the need for consistent definitions in replication studies, as others have highlighted.[32]

Collectively, therefore, our data provide further support for the observation that a SNP, which has not been associated with susceptibility to RA (MAF in 3879 UK RA cases: 0.103; MAF in 8428 controls: 0.104; OR: 0.994 (95% CI: 0.910-1.085), P = 0.89[2]) is associated with disease outcome. This highlights possible new directions for candidate gene or pathway-based studies in complex disease genetics, and suggests that by identifying other genetic variants that associate with outcome, it may ultimately be possible to develop prognostic tests that have sufficient performance to guide treatment decisions. As with studies of disease susceptibility, however, such work will probably require large sample sizes and meta-analyses to confirm associations at genome-wide significance levels. International consortia will undoubtedly be critical in achieving this goal.

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Figure Legend

Figure 1. Loss of Foxo3 activity predisposes to more severe arthritis in a mouse model

(A-D) Collagen-induced arthritis was induced in littermate Foxo3+/+, Foxo3+/- and Foxo3-/- mice. Results representative of two experiments each with a minimum of 6 mice per group. Data are represented as Mean ± SEM. Wilcoxon signed-rank test unless indicated.
(A) Disease severity scores (sum of limb scores where each limb is scored from 0-4, per Brand et al. 2007). Two-way ANOVA.
(B) Light microscopy of H&E stained joint sections from Foxo3-/- and Foxo3+/+ mice demonstrating loss of joint space and synovial expansion. Day 14.
(C) Anti-collagen antibody titres (day 14). AU, Arbitrary Units.
(D) Serum IL-6 (day 14)